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Chromosome segregation is triggered by separase, an enzyme that cleaves cohesin, the protein complex that holds sister chromatids together. Separase activation requires the destruction of its inhibitor, securin, which occurs only upon the correct attachment of chromosomes to the spindle. However, other mechanisms restrict separase activity to the appropriate window in the cell cycle because cohesin is cleaved in a timely manner in securin-deficient cells. We investigated the mechanism by which the protector protein Shugoshin counteracts cohesin cleavage in budding yeast. We show that Shugoshin can prevent separase activation independently of securin. Instead, PP2A^{Cdc55} is essential for Shugoshin-mediated inhibition of separase. Loss of both securin and Cdc55 leads to premature sister chromatid separation, resulting in aneuploidy. We propose that Cdc55 is a separase inhibitor that acts downstream from Shugoshin under conditions where sister chromatids are not under tension.

[*Keywords:* PP2A; Shugoshin; separase; cohesion; mitosis; meiosis]

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The separation of chromosomes during cell division is an irreversible event that must be tightly controlled to safeguard against aneuploidy. Sister kinetochores attach to microtubules from opposite spindle pole bodies at metaphase in preparation for their segregation during anaphase of mitosis. The cohesin complex facilitates this process by linking sister chromatids, thereby resisting opposing microtubule forces to generate tension at kinetochores. Once all sister chromatids have made proper bipolar attachments, cohesin is abruptly destroyed, due to cleavage of its Scc1/Mcd1/Rad21 subunit by a protease known as separase, thereby triggering chromosome segregation (Nasmyth 2002).

Separase must be exquisitely controlled. An inhibitory chaperone, known as securin, plays a key role in preventing separase activation (Ciosk et al. 1998; Uhlmann et al. 1999; Hornig et al. 2002; Waizenegger et al. 2002). Securin is destroyed at the onset of anaphase owing to its ubiquitination by the anaphase-promoting complex (APC), coupled to its activator, Cdc20 (Cohen-Fix et al. 1996; Funabiki et al. 1996b; Zou et al. 1999; Peters 2006), thereby liberating separase. The spindle assembly checkpoint (SAC) prevents securin ubiquitination in response to defective kinetochore–microtubule attachments by inhibiting APC^{Cdc20} (May and Hardwick 2006).

Despite the importance of securin in preventing separase activation, it is clear that other mechanisms exist to restrict cohesin cleavage to the appropriate window in the cell cycle. Although fission yeast and *Drosophila* securins are essential for viability (Funabiki et al. 1996a; Stratmann and Lehner 1996), budding yeast cells lacking securin are viable and initiate anaphase in a timely manner (Alexandru et al. 1999). Similarly, *securin*-deficient mice appear normal and mammalian cells exhibit only mild or transient phenotypes in the absence of securin (Jallepalli et al. 2001; Mei et al. 2001; Wang et al. 2001; Pfleghaar et al. 2005). An additional level of separase regulation is indeed achieved in vertebrate cells by the inhibitory phosphorylation-dependent binding of Cdk1/cyclin B1 (Stemmann et al. 2001; Gorr et al. 2005).

In addition to this temporal control, cohesin cleavage is additionally subject to spatial regulation during meiosis (Marston and Amon 2004). Separase-dependent cleavage of the meiosis-specific Scc1 homolog, Rec8, occurs on chromosome arms during meiosis I but is prevented in the vicinity of centromeres until meiosis II owing to the Shugoshin/Mei-S332 family of protector proteins (Kerrebrock et al. 1995; Katis et al. 2004; Kitajima et al. 2004; Marston et al. 2004; Rabitsch et al. 2004). Shugoshin (Sgo1) achieves this, at least in part, through recruitment of the protein phosphatase 2A, coupled to

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its B′ regulatory subunit (Rts1 in budding yeast) to centromeres (Kitajima et al. 2006; Riedel et al. 2006; Tang et al. 2006). $PP2A^{Rts1}$ is thought to maintain Rec8 around centromeres in its unphosphorylated state, making it refractory to cleavage by separase (Brar et al. 2006; Kitajima et al. 2006; Riedel et al. 2006). However, prevention of Rec8 phosphorylation either by Cdc5 depletion or mutation of its phosphorylation sites blocks cohesin cleavage only in the presence of Sgo1 (Brar et al. 2006). This suggests that Sgo1 prevents cohesin cleavage in ways other than inhibiting cohesin phosphorylation. Shugoshins also contribute to accurate chromosome segregation during mitosis by monitoring and promoting sister kinetochore biorientation (Indjeian et al. 2005; Indjeian and Murray 2007; Kawashima et al. 2007; Vanoosthuyse et al. 2007; Kiburz et al. 2008).

The centromeric cohesin protection and sister kinetochore biorientation functions of Shugoshins all culminate in the restraint of separase activity. We sought to learn more about the pathways by which Shugoshins accomplish their functions in cell division. Budding yeast has a single Shugoshin, *SGO1*, that both protects centromeric cohesin during meiosis I and monitors the biorientation of sister chromatids during mitosis. We show here that Sgo1 can inhibit separase activity independently of securin. $PP2A^{Cdc55}$ acts downstream from Sgo1 to fully inhibit separase and both monitors sister kinetochore biorientation and prevents cohesin cleavage during meiosis. Cells lacking both securin and Cdc55 prematurely separate sister chromatids, giving rise to aneuploidy. We propose that Cdc55 is a separase inhibitor that is activated by Sgo1 when sister chromatids are not under tension.

Results

Overproduced Sgo1 inhibits anaphase onset

To address the mechanism of Sgo1 function we developed an assay based on its overproduction. Wild-type cells and cells carrying multiple copies of *SGO1* under control of the galactose-inducible promoter (*pGAL*) were released from a G1 block synchronously into the cell cycle in the presence of galactose to induce *SGO1* overexpression. Wild-type cells progressed synchronously through metaphase and entered anaphase, as judged by spindle morphology (Fig. 1A) and timely degradation of the anaphase inhibitor securin (Pds1 in budding yeast) (Fig. 1B). In contrast, cells overexpressing *SGO1* exhibited a pronounced metaphase delay (Fig. 1A,B). For ease of interpretation of spindle morphology data in this and subsequent experiments we calculated the overall ratio of metaphase: anaphase spindles throughout the time course. Note that this value is close to 1 for the wild type, but increased in the *SGO1*-overexpressing cells, indicating a metaphase delay (Fig. 1A, boxes).

During metaphase, endogenous Sgo1 associates with an ~50-kb cohesin-enriched region surrounding centromeres (the pericentromere) (Kiburz et al. 2005). Chromatin immunoprecipitation (ChIP) revealed that overproduced Sgo1 localized not only to this region but also along the length of chromosomes (Supplemental Fig. S1A–C). Association of endogenous Sgo1 with the pericentromere depends on the kinetochore protein, Bub1 (Kiburz et al. 2005; Riedel et al. 2006; Fernius and Hardwick 2007) and we found that *bub1Δ* also reduced the amount of overproduced Sgo1 on chromosomes (Supplemental Fig. S1B,C), but not its overall levels (Supplemental Fig. S2). The failure of overproduced Sgo1 to accumulate on chromatin correlated with an inability to block anaphase onset. In *bub1Δ pGAL-SGO1* cells, Pds1 destruction and the appearance of anaphase spindles occurred with a timing similar to that seen in *bub1Δ* cells (Supplemental Fig. S1D,E). Therefore, *BUB1* is required for the metaphase delay caused by *SGO1* overexpression, perhaps due to a requirement for Sgo1 to be associated with chromatin.

SGO1 *overexpression causes a metaphase delay independently of spindle checkpoint components, Aurora kinase, and securin*

In addition to its role in localizing Sgo1, Bub1 is a component of the SAC. However, the metaphase delay induced by *SGO1* overexpression does not require another SAC component, *MAD1* (Supplemental Fig. S1F,G). Similarly, a functional Aurora kinase (*IPL1*), which destabilizes inappropriate microtubule–kinetochore attachments, is not required for the metaphase delay induced by *SGO1* overexpression (Supplemental Fig. S3). These findings raised the possibility that securin might also be dispensable for the delay in anaphase onset in *pGAL-SGO1* cells and, indeed, we found this to be the case (Fig. 1C,D). Taken together, these results indicate that *SGO1* prevents anaphase onset through a mechanism that is independent of securin and the canonical SAC.

Separase is inhibited upon SGO1 *overexpression*

Since *SGO1* overexpression causes a metaphase delay in the absence of securin, we reasoned that either separase is inhibited independently of securin, or that its substrate, the cohesin protein Scc1, is made resistant to separase-dependent proteolysis. Both these possibilities predict that cleavage of Scc1 is inhibited upon *SGO1* overexpression and indeed we found this to be the case. In wild-type and *pds1Δ* cells, the levels of full-length Scc1 decreased simultaneously with the appearance of anaphase spindles and a shorter Scc1 cleavage fragment (Fig. 1C,D). In cells overexpressing *SGO1*, however, this decline in full-length Scc1 and appearance of cleavage product was greatly retarded, even in the absence of *PDS1* (Fig. 1C,D). To test whether separase was inhibited we examined the cleavage of its other known substrate, the kinetochore protein, Slk19 (Fig. 1E,F; Sullivan et al. 2001). Slk19 exists in multiple forms, the two faster migrating bands of which correspond to phosphorylated and unphosphorylated cleavage product and are relatively stable (Fig. 1E,F). The slower migrating species is full-length Slk19 that, in wild-type cells and *pds1Δ* cells, is maximally phosphorylated at metaphase before declining at anaphase onset (Fig. 1E,F). In cells overexpressing *SGO1*, however, full-length Slk19 remains stabilized,

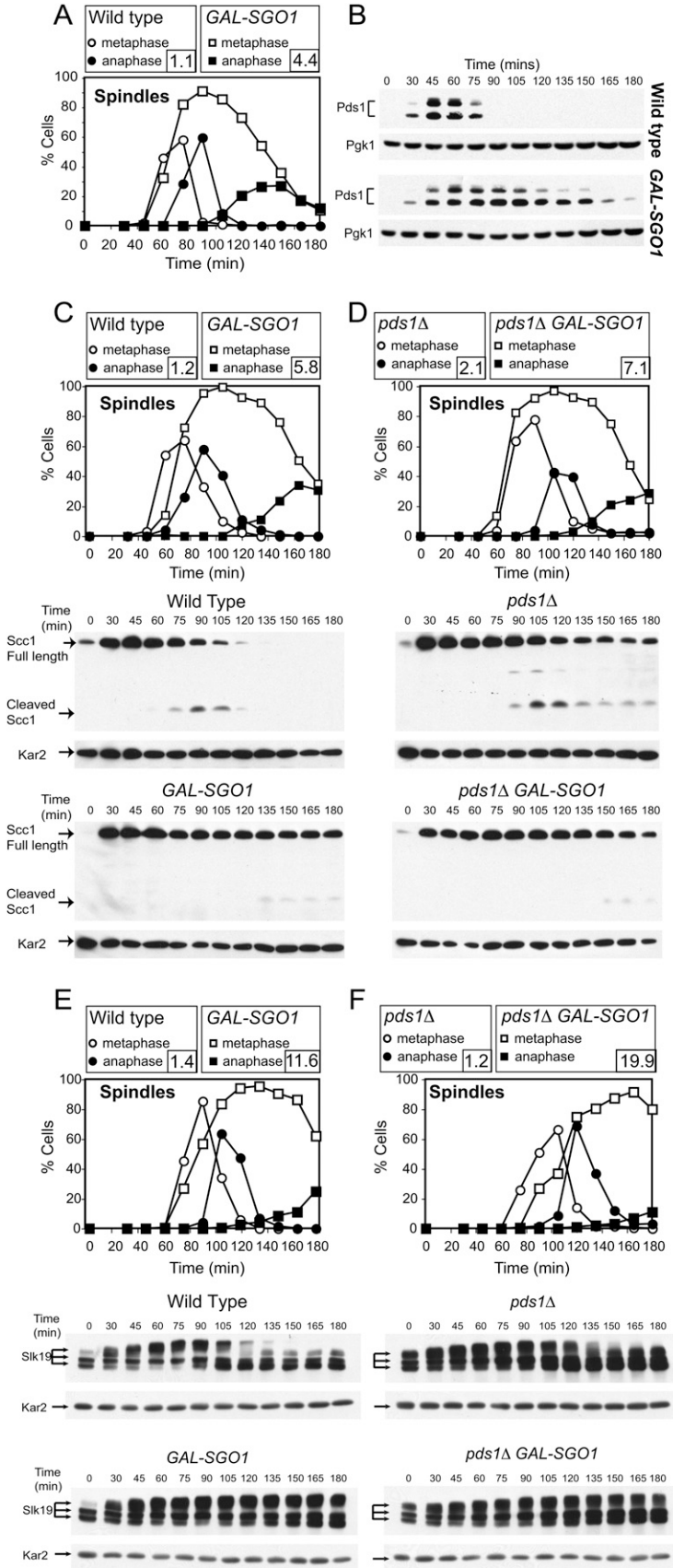

Figure 1. *SGO1* overexpression inhibits separase activity in a *PDS1*-independent manner. Overexpression of *SGO1* causes a metaphase delay. Cells were arrested in G1 in raffinose medium (YEPR) using α-factor, preinduced with galactose for 30 min, and released into medium containing both raffinose and galactose (YEPRG). α-Factor was added again after release to limit our analysis to a single cell cycle. Samples were taken at the indicated time points for immunoblot analysis and to determine percentages of cells with short (metaphase) and long (anaphase) spindles after tubulin immunoflurescence (at least 200 cells per time point). The numbers in boxes correspond to the overall ratio of metaphase to anaphase cells throughout the time course for each strain. Pgk1 and Kar2 are shown as loading controls. (*A*,*B*) Pds1 is stabilized upon *SGO1* overexpression. Spindle morphology (*A*) and Pds1-3HA protein levels (*B*) of wild-type (AM1290) and *pGAL-SGO1* (AM3917) cells carrying *PDS1-3HA*. (*C*,*D*) *SGO1* overexpression inhibits cleavage of Scc1. (*C*,*D*) Spindle morphology (*top* panels) and anti-HA immunoblot (*bottom* panels) of wild-type (AM1145), *pGAL-SGO1* (AM1126), *pds1Δ* (AM1127), and *pds1Δ pGAL-SGO1* (AM1128) cells all carrying *SCC1-6HA*. (*E*,*F*) Slk19 cleavage is inhibited by *SGO1* overexpression. Spindle morphology (*top* panels) and anti-Myc immunoblot (*bottom* panels) of wild-type (AM2753), *pGAL-SGO1* (AM1066), *pds1Δ* (AM3298), and *pds1Δ pGAL-SGO1* (AM1604) cells all carrying *SLK19-13MYC*.

even in the absence of *PDS1* (Fig. 1E,F). Therefore, *SGO1* overexpression inhibits cleavage of both known separase substrates in a securin-independent manner.

Cohesin cleavage is sufficient to allow spindle elongation in SGO1*-overexpressing cells*

We reasoned that, if prevention of cohesin cleavage due to separase inhibition is responsible for the metaphase delay in *SGO1*-overexpressing cells, forced cleavage of cohesin should trigger anaphase onset. We used a version of Scc1 with one of its two separase cleavage sites exchanged for the recognition sequence for TEV protease (*SCC1-TEV*), the ectopic cleavage of which can be induced by expression of the gene encoding TEV protease from the *pGAL* promoter (*pGAL-TEV*) (Uhlmann et al. 2000). Supplemental Fig. S4 shows that artificial cleavage of cohesin abolished the metaphase delay caused by *SGO1* overexpression. Anaphase spindles tended to be shorter in these cells (data not shown), consistent with a failure to activate separase (Higuchi and Uhlmann 2005; see also below). We conclude that cohesin cleavage is sufficient to allow anaphase onset in *SGO1*-overexpressing cells.

Sgo1 can inhibit the nonproteolytic function of separase

In addition to its role as a protease, a nonproteolytic function of separase initiates mitotic exit as part of the Cdc14 early anaphase release (FEAR) network, which promotes release of the phosphatase Cdc14 from the nucleolus during early anaphase (Fig. 2A; for review, see Stegmeier and Amon 2004). To test whether this nonproteolytic role of separase was also inhibited upon *SGO1* overexpression, we analyzed the timing of Cdc14 release from the nucleolus and compared it with spindle length in cells lacking *PDS1* to rule out possible inhibition of separase due to securin stabilization. In *pds1Δ* cells, as expected, Cdc14 was released from the nucleolus into the nucleus and cytoplasm only as spindles became longer than 2 μm (Fig. 2B–D). In contrast, Cdc14 release from the nucleolus was inhibited in *pds1Δ* cells overexpressing *SGO1* (Fig. 2B). Since Cdc14 is not normally released from the nucleolus in cells with a short spindle, the predominant class in *SGO1*-overexpressing cells, we further examined Cdc14 localization in cells lacking cohesin, which allows spindle elongation even if separase is inhibited (Supplemental Fig. S4). Depletion of *SCC1* by its placement under the control of the methionine-repressible *pMET3* promoter caused an increase in spindle length in *SGO1*-overexpressing cells (Fig. 2D, cf. *pds1Δ pMET-SCC1 pGAL-SGO1* and *pds1Δ pGAL-SGO1*), although Cdc14 release was still inhibited (Fig. 2B). These results indicate that *SGO1* overexpression impairs Cdc14 release from the nucleolus in a Pds1-independent manner. Again, elongated spindles tended to be shorter in cohesin-depleted cells where *SGO1* was overexpressed (Fig. 2D, cf. *pds1Δ pMET-SCC1* and *pds1Δ pMET-SCC1 pGAL-SGO1*). Because separase-dependent Cdc14 activation is required for full spindle elongation (Higuchi and Uhlmann 2005), this further suggests that separase is inhibited upon *SGO1* overexpression. In support of this interpretation, *ESP1* overexpression can overcome the metaphase delay caused by high levels of Sgo1 (Fig. 2E).

PP2A^{Cdc55} is required for inhibition of anaphase onset upon SGO1 *overexpression*

Our results indicate that *SGO1* overexpression causes separase inhibition and a metaphase arrest independently of securin. How might this separase inhibition occur? A candidate downstream effector of Sgo1 in separase inhibition is the protein phosphatase 2A (PP2A), a tripartite complex of scaffold (A), regulatory (B), and catalytic (C) subunits. In budding yeast, there are two regulatory subunits, Cdc55 (B) and Rts1 (B′). During meiosis, Sgo1 recruits PP2A coupled to its B′ regulatory subunit (Rts1 in budding yeast) to centromeric regions and cohesin is protected locally likely as a result of its dephosphorylation by PP2A^{Rts1}, which makes it a poor substrate for cleavage by separase (Lee and Amon 2003; Brar et al. 2006; Kitajima et al. 2006; Riedel et al. 2006). PP2A coupled to the B type regulatory subunit, Cdc55, is an inhibitor of mitotic exit and, notably, forms a complex with separase (Minshull et al. 1996; Queralt et al. 2006; Wang and Ng 2006; Yellman and Burke 2006). We therefore tested the requirement for *RTS1* and *CDC55* in the block to anaphase onset caused by *SGO1* overexpression. Deletion of *RTS1* only slightly advanced the onset of anaphase in *SGO1*-overexpressing cells (Fig. 3A,B). However, strikingly, deletion of *CDC55* almost completely rescued the metaphase delay of *SGO1*-overexpressing cells (Fig. 3A,C). The slight delay in anaphase onset in *cdc55Δ pGAL-SGO1* cells as compared with *cdc55Δ* cells (Fig. 3C), together with the modest advancement of anaphase in *SGO1*-overexpressing cells lacking *RTS1* (Fig. 3B), could represent a minor role for PP2A^{Rts1} in Scc1 dephosphorylation, as reported for Rec8 in meiosis (Kitajima et al. 2006; Riedel et al. 2006). However, we were not able to test whether deletion of both *RTS1* and *CDC55* completely abolished the metaphase delay of *SGO1*-overexpressing cells due to the severely impaired growth of the *rts1Δ cdc55Δ* double mutant (data not shown).

To investigate whether the *CDC55*-dependent block to anaphase onset was mediated through PP2A, we examined the ability of *SGO1* overexpression to induce a metaphase delay in cells where the PP2A catalytic subunits are mutated (*pph21-L369Δ pph22Δ*) such that Cdc55 association with the PP2A holoenzyme is prevented (Evans and Hemmings 2000). Figure 3D shows that *SGO1* overexpression did not induce a metaphase delay in *pph21-L369Δ pph22Δ* cells. We also found that neither the amount of overproduced Sgo1 (Supplemental Fig. S2) nor its association with chromosomes (Fig. 3E,F) were affected by deletion of *CDC55*. We conclude that PP2A^{Cdc55} acts downstream from *SGO1* overexpression in preventing cell cycle progression.

CDC55 *deletion allows separase activation in* SGO1*-overexpressing cells*

We tested whether *CDC55* was required for the stabilization of Pds1 caused by *SGO1* overexpression. Deletion

of *CDC55* reduced, but did not abolish, the delay in Pds1 degradation in *SGO1*-overexpressing cells (Fig. 3G,H). We next monitored the cohesin, Scc1, by Western blotting to determine whether its separase-dependent cleavage was coupled to anaphase onset in *SGO1*-overexpressing cells lacking *CDC55*. For ease of detection, we examined cohesin cleavage in *ubr1Δ* cells, in which the Scc1 cleavage fragment is stabilized (Rao et al. 2001). As before, cleaved Scc1 accumulated in wild-type cells concomitant with the disappearance of full-length Scc1 and appearance of anaphase spindles (Fig. 4A,B) but in cells overexpressing *SGO1*, both Scc1 cleavage and the appearance of anaphase spindles (Fig. 4A,C) were delayed. Both the decline in full-length Scc1 and the appearance of its cleavage fragment were also modestly delayed in *ubr1Δ cdc55Δ* cells, consistent with the slower entry into anaphase of these cells (Fig. 4D,E). Surprisingly, Scc1 levels declined slowly in *ubr1Δ cdc55Δ pGAL-SGO1* cells, although a Scc1 cleavage product accumulated around the time at which anaphase spindles appeared (75 min) (Fig. 4D,F) and with a timing similar to that of *ubr1Δ cdc55Δ* cells (Fig. 4D,E). These findings suggested that *cdc55Δ* advances anaphase in *pGAL-SGO1* cells without complete Pds1 degradation and while allowing only a small subset of Scc1 to be cleaved.

To ask if the uncleaved Scc1 in *SGO1*-overexpressing cells lacking *CDC55* was associated with chromatin during anaphase, we examined Scc1 localization on chromosome spreads. Figure 4, G–K, shows that Scc1 dissociated from chromosomes concomitant with the onset of anaphase spindles in wild-type and *cdc55Δ* cells whether *SGO1* was overexpressed or not. Therefore, the persistent full-length Scc1 in *SGO1*-overexpressing cells lacking *CDC55* cannot hold sister chromatids together. Consistent with these results, deletion of *CDC55* in *SGO1*-overexpressing cells allows for a normal anaphase with sister chromatids segregating to opposite poles (Supplemental Fig. S5).

To test directly whether cohesin cleavage is required for anaphase onset in *SGO1*-overexpressing cells lacking *CDC55*, we used a version of Scc1 that has had its recognition sites for separase mutated and cannot be

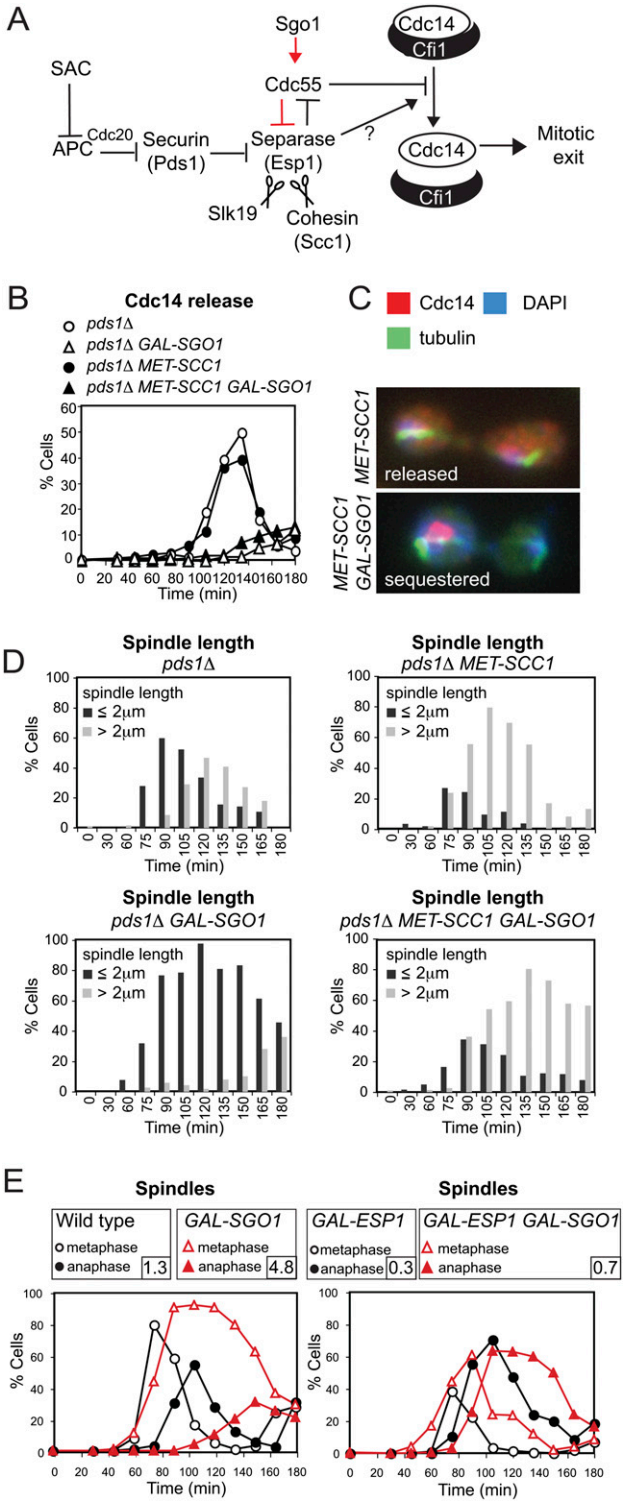

Figure 2. *SGO1* overexpression inhibits the nonproteolytic function of separase in mitotic exit. (*A*) Schematic diagram showing factors involved in chromosome segregation and mitotic exit. See text for details. Red arrows indicate the present findings. (*B–D*) Cdc14 release from the nucleolus and full spindle elongation is inhibited by *SGO1* overexpression. Cells of strains *pds1Δ* (AM3509), *pds1Δ pGAL-SGO1* (AM3510), *pds1Δ pMET-SCC1* (AM4435), and *pds1Δ pMET-SCC1 pGAL-SGO1* (AM4436) all carrying *3HA-CDC14* were grown in medium lacking methionine and containing raffinose (SC/−Met/R). Cells were transferred to YEPR + 8 mM methionine and arrested in G1, preinduced with galactose for 30 min, and then released into YEPRG + 8 mM methonine. Percentages of cells with 3HA-Cdc14 released from the nucleolus (*B*) and examples (*C*) after immunofluoresence. (*D*) Measurements of spindle length at the indicated time points from images of tubulin staining. Spindle lengths are divided into two classes, the first representing spindles ≤2 μM, typical of cells in metaphase and the second representing spindles >2 μM. (*E*) Co-overexpression of *ESP1* advances anaphase onset in *SGO1*-overexpressing cells. Wild type (AM1176), *pGAL-SGO1* (AM870), *pGAL-ESP1* (AM3225), and *pGAL-ESP1 pGAL-SGO1* (AM1667) were grown as described in Figure 1 and spindle morphology analyzed.

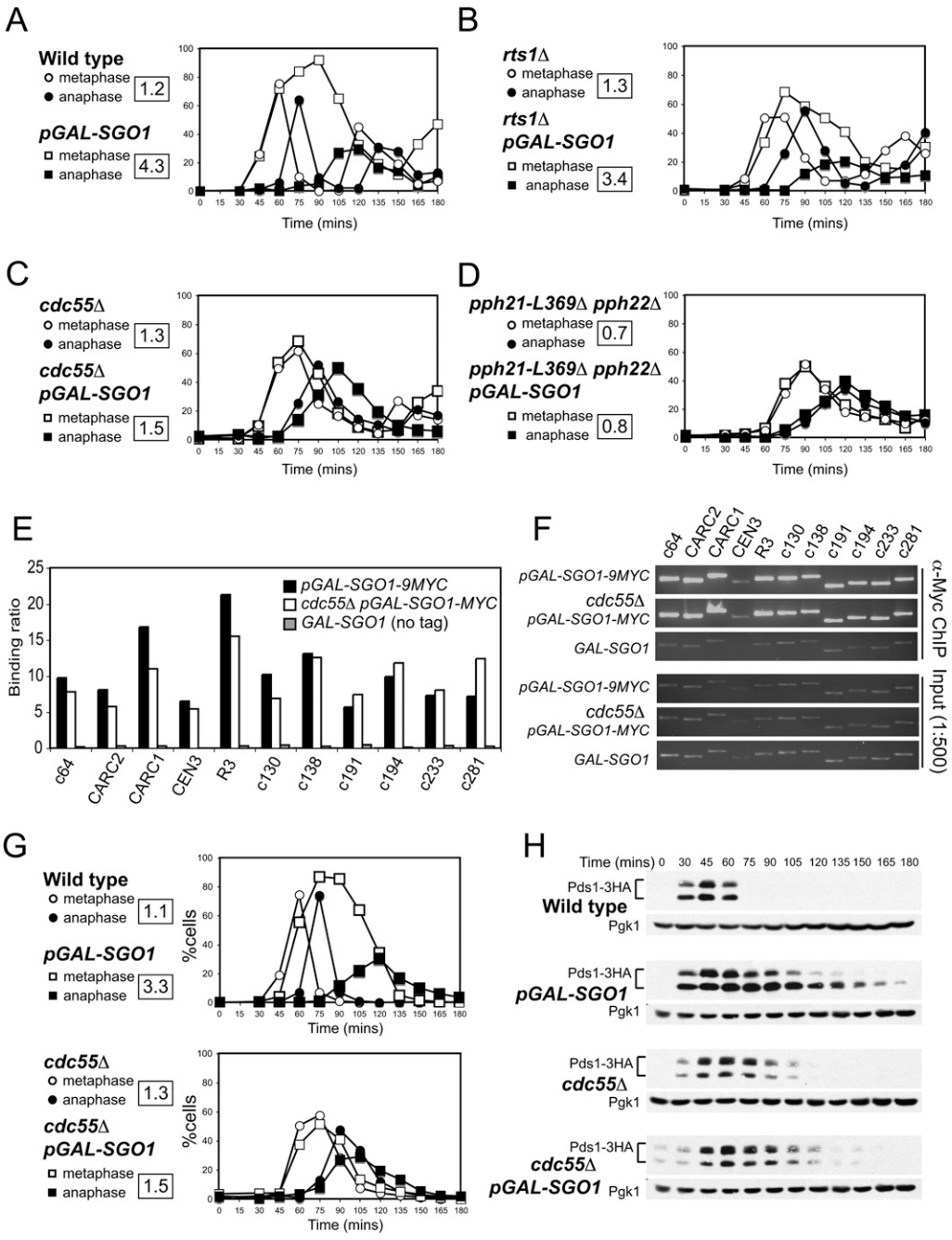

Figure 3. PP2A^{Cdc55} is required to prevent spindle elongation and securin degradation in *SGO1*-overexpressing cells. (*A–D*) *SGO1* overexpression causes a metaphase delay in otherwise wild-type (*A*) or *rts1Δ* (*B*) cells, but not *cdc55Δ* (*C*) and *pph21-L369Δ pph22Δ* (*D*) cells. Wild-type (AM1176), *pGAL-SGO1* (AM870), *rts1Δ* (AM3209), *rts1Δ pGAL-SGO1* (AM3306), *cdc55Δ* (AM3164), *cdc55Δ pGAL-SGO1* (AM3239), *pph21-L369Δ pph22Δ* (AM5271), *pph21-L369Δ pph22Δ pGAL-SGO1* (AM5269) strains were treated and analyzed as described in Figure 1 except that they were grown at 30°C and not retreated with α-factor. (*E*,*F*) Deletion of *CDC55* does not impair the association of overproduced *SGO1* with chromatin. Binding ratios (*E*) determined after PCR analysis (*F*) of ChIP samples using primers to sites at the centromere (CEN3), pericentromere (CARC2, CARC1, R3, c130), or arms (c64, c138, c191, c194, c233, c281) of chromosome III (see Supplemental Fig. S1A). Strains carrying *pGAL-SGO1-MYC* and otherwise wild type (AM1392) or *cdc55Δ* (AM4843) compared with a no-tag control (AM870) were grown at 30°C and analyzed as described in Supplemental Figure S1. (*G*,*H*) Deletion of *CDC55* advances securin degradation in *SGO1*-overexpressing cells. Wild-type (AM1290), *pGAL-SGO1* (AM3917), *cdc55Δ* (AM4432), and *pGAL-SGO1 cdc55Δ* (AM4431) cells were grown at 30°C but otherwise as described in Supplemental Figure S1.

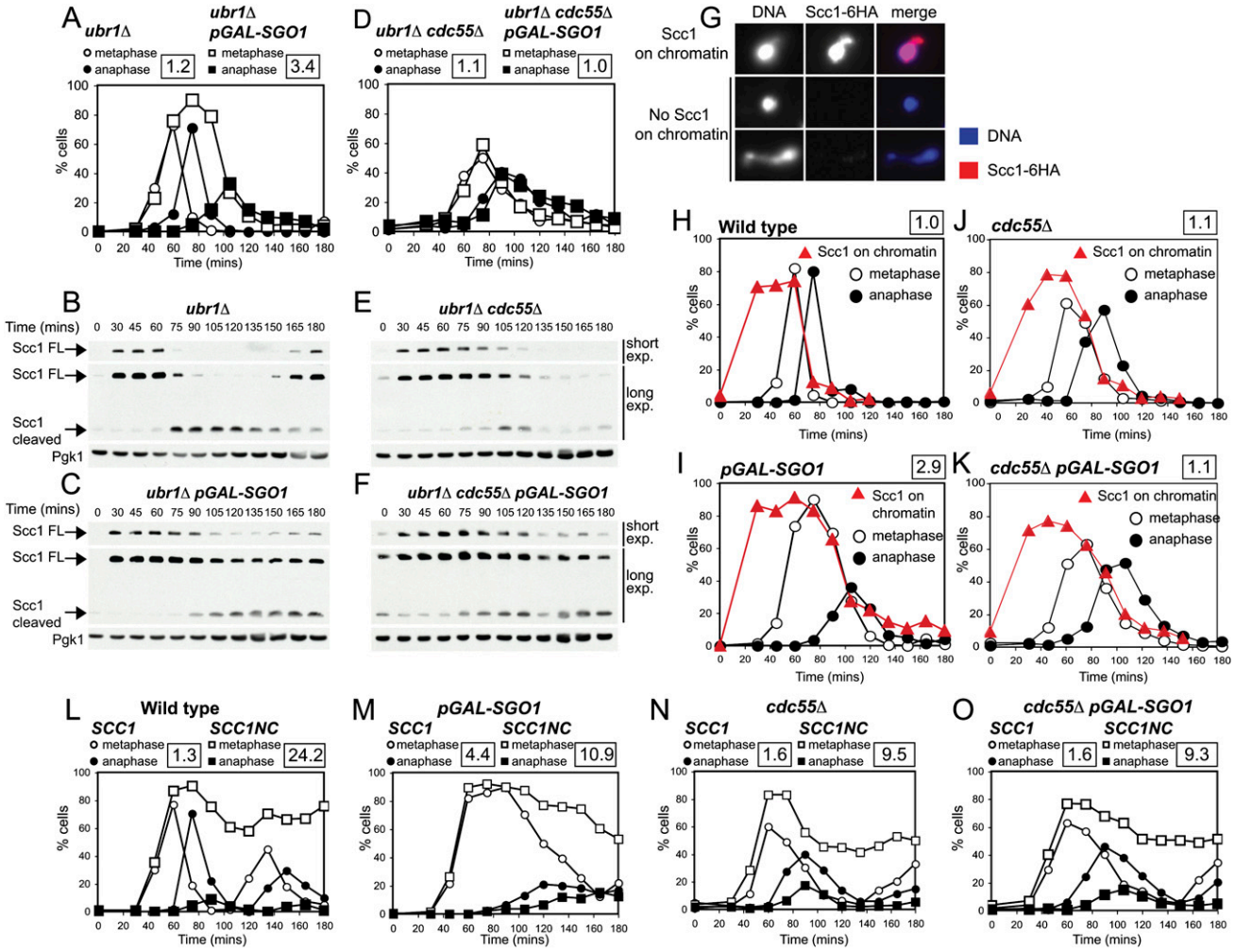

Figure 4. Cohesin cleavage is incomplete, but essential for anaphase onset, in *cdc55Δ* cells overexpressing *SGO1*. (*A–K*) Cohesin is cleaved inefficiently in *SGO1*-overexpressing cells lacking *CDC55* but is removed from chromosomes. Spindle morphology (*A*,*D*) and immunoblot of Scc1 protein levels (*B*,*C*,*E*,*F*) were examined in cells grown at 30°C and analyzed as described in Figure 1. Strains all carried a *SCC1-6HA* fusion, were *ubr1Δ*, and were otherwise wild type (AM4576), *pGAL-SGO1* (AM4575), *cdc55Δ* (AM4574), or *cdc55Δ pGAL-SGO1* (AM4611). (*G–K*) Analysis of Scc1 localization on chromosome spreads of cells of wild type (AM1145), *cdc55Δ* (AM4757), *pGAL-SGO1* (AM1126), and *cdc55Δ pGAL-SGO1* (AM4758) treated as described above. (*G*) Examples of Scc1 staining. (*H–K*) Percentages of cells with detectable Scc1 on chromatin as well as metaphase and anaphase spindles are shown for the indicated genotypes. (*L–O*) Cohesin cleavage is required for anaphase onset in *SGO1*-overexpressing cells lacking *CDC55*. Strains of wild type (AM1176), *pGAL-SCC1-NC* (AM4564) (*L*); *pGAL-SGO1* (AM870), *pGAL-SGO1 pGAL-SCC1-NC* (AM5306) (*M*); *cdc55Δ* (AM3164), *cdc55Δ pGAL-SCC1-NC* (AM5169) (*N*); and *cdc55Δ pGAL-SGO1* (AM3239); *cdc55Δ pGAL-SGO1 pGAL-SCC1-NC* (AM4620) (*O*) were grown at 30°C as described in Figure 1, but were not retreated with α-factor, and the percentages of metaphase and anaphase spindles were scored.

cleaved (Uhlmann et al. 1999). Expression of this non-cleavable form of cohesin from the *pGAL* promoter (*pGAL-SCC1-NC*) in otherwise wild-type, *pGAL-SGO1*, or *cdc55Δ* cells caused a strong metaphase delay (Fig. 4L–N). Similarly, Scc1-NC inhibited spindle elongation in *SGO1*-overexpressing cells lacking *CDC55* (Fig. 4O). Additionally, we found that a functional separase is essential for spindle elongation in *cdc55Δ pGAL-SGO1* cells (Supplemental Fig. S6). Taken together, these findings demonstrate that *CDC55* deletion bypasses the metaphase delay caused by *SGO1* overexpression by allowing at least a subset of cohesin to be cleaved by separase.

CDC55 *acts downstream from* SGO1 *in separase inhibition*

Our findings suggested that *CDC55* may act downstream from *SGO1* in the inhibition of separase. Interestingly, Chiroli et al. (2007) found that overexpression of *CDC55* causes a delay in metaphase that is independent of securin. We confirmed these results and in addition found

that overexpression of *CDC55* gives a phenotype that is strikingly similar to that seen when overexpressing *SGO1*. That is, securin is stabilized in *CDC55*-overexpressing cells and the cleavage of separase substrates Scc1 and Slk19 is inhibited even in the absence of *PDS1* (Supplemental Fig. S7A–D). We found that *SGO1* is also not required for the metaphase delay caused by *CDC55* overexpression (Supplemental Fig. S7E,F). These results support the notion that Cdc55 acts downstream from Sgo1 to inhibit separase.

Sensitivity of cdc55Δ, sgo1Δ, *and* mad1Δ *mutants to microtubule-depolymerizing drugs*

Both *CDC55* and *SGO1* play poorly defined roles in the SAC and show reduced growth on the microtubule-depolymerizing drug, benomyl (Minshull et al. 1996; Wang and Burke 1997; Indjeian et al. 2005; Yellman and Burke 2006). We found that the *cdc55Δ* mutant is slightly more benomyl-sensitive than the *sgo1Δ* mutant but is not significantly further compromised in combination with it (Supplemental Fig. S8A). Both *cdc55Δ* and *sgo1Δ* mutants grow more poorly on benomyl than, and show additive effects with, the canonical SAC mutant, *mad1Δ*. However, *rts1Δ* does not display benomyl sensitivity. These results lend support to the idea that Sgo1 and Cdc55 function in a common checkpoint pathway that is distinct from the canonical SAC. We note, however, that Sgo1 and Cdc55 must also have distinct cellular roles, since the *cdc55Δ sgo1Δ* double mutant exhibits a more severe growth defect than either single mutant alone in the absence of microtubule-depolymerizing drugs (Supplemental Fig. S8A; D. Clift and A.L. Marston, unpubl.).

Negative regulation of mitotic exit by Sgo1 and Cdc55

The role of Cdc55 in the SAC has been attributed to its function in the inhibition of mitotic exit by ensuring the sequestration of Cdc14 in the nucleolus (Yellman and Burke 2006). A group of genes known as the FEAR network initiate Cdc14 release from the nucleolus and this is maintained by the mitotic exit network (MEN) (Stegmeier and Amon 2004). Cells lacking both the FEAR activator, *SPO12*, and the MEN activator, *LTE1*, are inviable (Stegmeier et al. 2002) but can be rescued by deletion of *CDC55*, indicating that Cdc55 negatively regulates mitotic exit (Yellman and Burke 2006). We found that *sgo1Δ spo12Δ lte1Δ* cells are also viable (Supplemental Fig. S8B), providing evidence that Sgo1 also inhibits mitotic exit and functions in a common pathway to Cdc55.

Cdc55 and Sgo1 respond to a lack of tension between sister kinetochores

We asked if the checkpoint defects of the *sgo1Δ* mutant are shared with *cdc55Δ*. Sgo1 is required to arrest the cell cycle in response to a lack of tension between sister kinetochores (Indjeian et al. 2005). To examine the requirement for *CDC55* in response to a lack of tension we depleted cohesin (using the *MET-SCC1* construct) and examined the stability of securin, Pds1, and the mitotic cyclin, Clb2, as well as the release of Cdc14 from the nucleolus. The absence of linkages between sister chromatids causes the stabilization of Pds1 and Clb2 and the sequestration of Cdc14 in the nucleolus in wild-type cells (Fig. 5A–C). However, the SAC mutant *mad1Δ* failed to arrest the cell cycle as evidenced by the destruction of both Pds1 and Clb2 and release of Cdc14 from the nucleolus. The *sgo1Δ* and *cdc55Δ* mutants also failed in cell cycle arrest but behaved in a manner that was distinct from the *mad1Δ* mutant: Cdc14 was released from the nucleolus, but both Pds1 and Clb2 appeared relatively stable on Western blots (Fig. 5A–C). Analysis of single cells, however, revealed that the disappearance of Pds1 was accelerated in a fraction of *sgo1Δ* and *cdc55Δ* cells (Fig. 5A). Therefore, the response of *sgo1Δ* and *cdc55Δ* mutants to a lack of tension between sister kinetochores is similar and characterized by inappropriate Cdc14 release from the nucleolus and partial degradation of Pds1.

Cdc55 prevents precocious separase activation during meiosis

Does Cdc55-dependent separase inhibition contribute to the centromeric cohesin protection function of Sgo1 during meiosis I? Sgo1 achieves this, in part, through the recruitment of $PP2A^{Rts1}$ to centromeres. $PP2A^{Rts1}$ is thought to counteract the Polo kinase-dependent

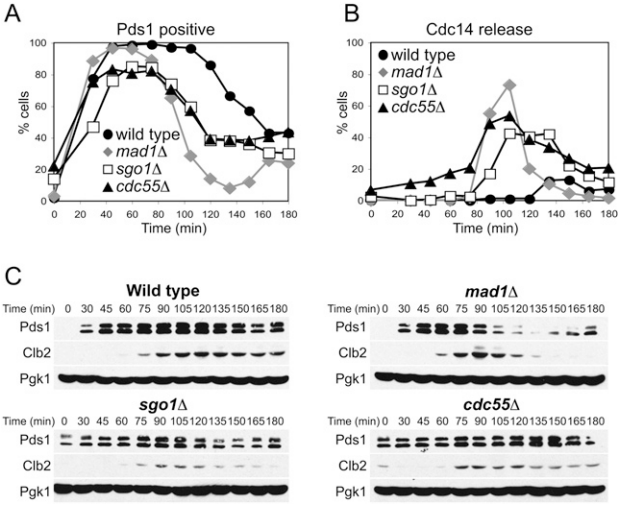

Figure 5. Tension-sensing defects in *sgo1Δ* and *cdc55Δ* mutants. (*A–C*) *SGO1* and *CDC55* are required for cell cycle arrest in response to a lack of tension. Strains carrying *pMET-SCC1*, and having *PDS1-18MYC* and *3HA-CDC14* fusions that were otherwise wild type (AM4772), *mad1Δ* (AM4856), *sgo1Δ* (AM4773), and *cdc55Δ* (AM4853), were grown as described in Figure 2 to deplete *SCC1* except the medium contained glucose rather than raffinose. (*A*,*B*) Percentages of cells at the indicated times after G1 release with (*A*) Pds1 staining or (*B*) Cdc14 released from the nucleolus on immunofluorescence samples. (*C*) Immunoblot analysis of Pds1-18Myc and Clb2 levels. Pgk1 is shown as a loading control.

phosphorylation of Rec8, thereby maintaining Rec8 in the unphosphorylated state and a poor substrate for separase-dependent cleavage. However, the inhibition of Rec8 phosphorylation either by depletion of Polo kinase (*CDC5* in budding yeast) or by using a Rec8 phosphorylation site mutant (*REC8-17A*) prevents cohesin cleavage only in the presence of Sgo1 (Brar et al. 2006). Therefore, Sgo1 prevents Rec8 cleavage through a mechanism other than reversing Cdc5-dependent phosphorylation. To ask if Cdc55 could be part of this mechanism, we examined Rec8 cleavage in cells carrying *CDC5* under control of the mitosis-specific *CLB2* promoter, resulting in its repression during meiosis (*pCLB2-CDC5*) (Lee and Amon 2003). In wild-type cells, the appearance of the Rec8 cleavage product coincided with a decrease in mononucleate cells (Fig. 6A,C). As described previously, Rec8 was underphosphorylated in *pCLB2-CDC5* cells, its cleavage was precluded (Fig. 6D, cf. wild type and *pCLB2-CDC5*), and nuclear division did not occur (Fig. 6B). Depletion of *SGO1* (using a *pCLB2-SGO1* allele) allows increased cohesin cleavage in *pCLB2-CDC5* cells and mononucleate cells declined in lieu of cells with bilobed, stretched, or divided nuclei (Fig. 6E,F; Brar et al. 2006). Note that cohesin cleavage is not expected to allow complete nuclear division in *pCLB2-CDC5* cells, owing to the persistence of unresolved Holliday junctions (Clyne et al. 2003). To investigate the importance of *CDC55* in averting the cleavage of underphosphorylated Rec8, we first induced *cdc55Δ* mutants to undergo meiosis; however, they were severely impaired in meiotic progression (data not shown). We therefore generated a conditional allele by placing *CDC55* under the control of the *CLB2* promoter (*pCLB2-CDC55*), which led to at least a partial depletion of *CDC55* during meiosis (Fig. 6G,H). *pCLB2-CDC55* mutants are impaired in meiotic progression and undergo a single nuclear division, suggesting that *CDC55* performs multiple functions during meiosis that are beyond the scope of the present study (data not shown). Nevertheless, Figure 6, B and H, shows that depletion of *CDC55* allows similar levels of Rec8 cleavage and a comparable reduction of mononucleate cells in *pCLB2-CDC5* cells as does *SGO1* depletion. Therefore, both *SGO1* and *CDC55* are required to shield underphosphorylated Rec8 from separase-dependent cleavage, indicating that Sgo1 and Cdc55 have overlapping functions in separase regulation in meiosis as well as mitosis.

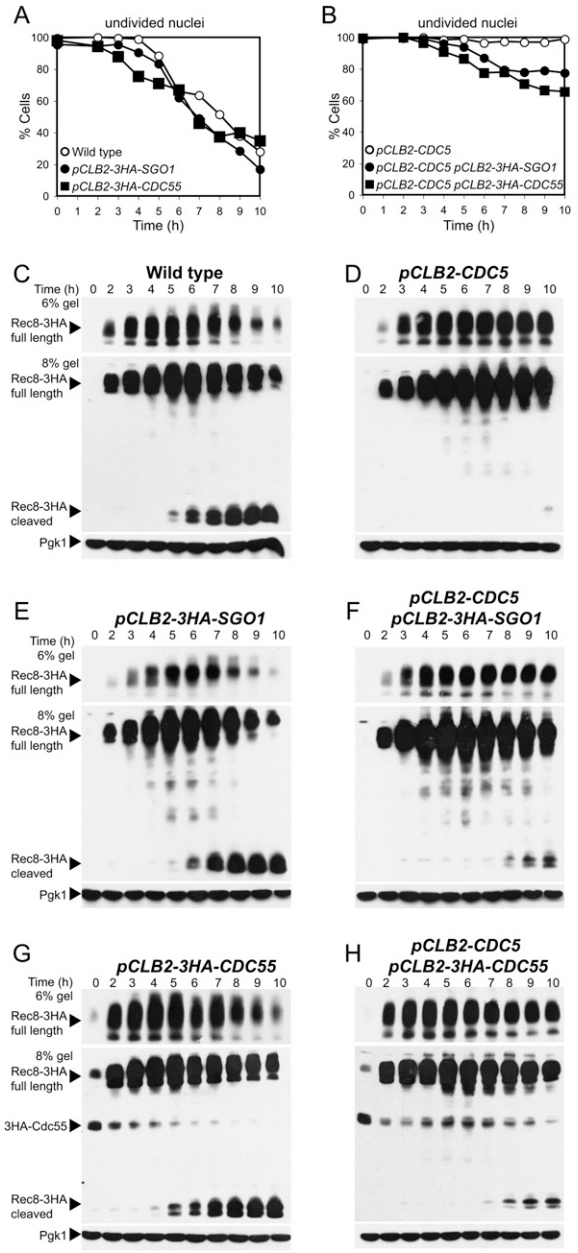

Figure 6. Depletion of *CDC55* partially alleviates the need for *CDC5* in Rec8 cleavage. Sporulation was induced in diploid cells of wild-type (AM4850), *pCLB2-CDC5* (AM4851), *pCLB2-3HA-SGO1* (AM4838), *pCLB2-CDC5 pCLB2-3HA-SGO1* (AM4800), *pCLB2-3HA-CDC55* (AM4938), and *pCLB2-CDC5 pCLB2-CDC55* (AM4937) cells and the percentage of undivided nuclei (*A*,*B*) determined and levels of Rec8-HA protein analyzed (*C*–*H*). Samples from the same experiment were run on 6% (*top*) and 8% (*bottom*) gels to allow visualization of the Rec8 phosphorylation shift and cleavage product, respectively. The positions of full-length Rec8-3HA (*C*–*H*), cleaved Rec8-3HA (*C*–*H*), and 3HA-Cdc55 (*G*,*H*) are shown. Pgk1 is shown as a loading control.

Cdc55 is essential for separase inhibition in the absence of securin

If Cdc55 is important for separase inhibition in a normal cell cycle, it would become essential in the absence of securin, a well-established separase inhibitor. Indeed, cells lacking both *CDC55* and *PDS1* are inviable (Tang and Wang 2006; Chiroli et al. 2007; data not shown). To examine the terminal phenotype of these cells, we constructed a conditional allele of *PDS1* by placing it under the methionine-repressible promoter, *pMET3*. As expected, a *cdc55Δ pMET-PDS1* strain failed to grow in the presence, but not the absence, of methonine (Supplemental Fig. S9). We examined the consequences of loss of both Cdc55 and Pds1 over a single cell cycle. Wild-type,

cdc55Δ, *pMET-PDS1*, and *cdc55Δ pMET-PDS1* cells, all carrying an Scc1-6HA fusion, were released from a G1 arrest in the presence of methonine, and cleavage of Scc1 was monitored as a marker for separase activity (Fig. 7A–D). In wild-type and *PDS1*-depleted cells, full-length Scc1 declined and a shorter cleavage fragment appeared simultaneously with spindle elongation (60 min) (Fig. 7A,B). Similarly, an Scc1 cleavage fragment appeared

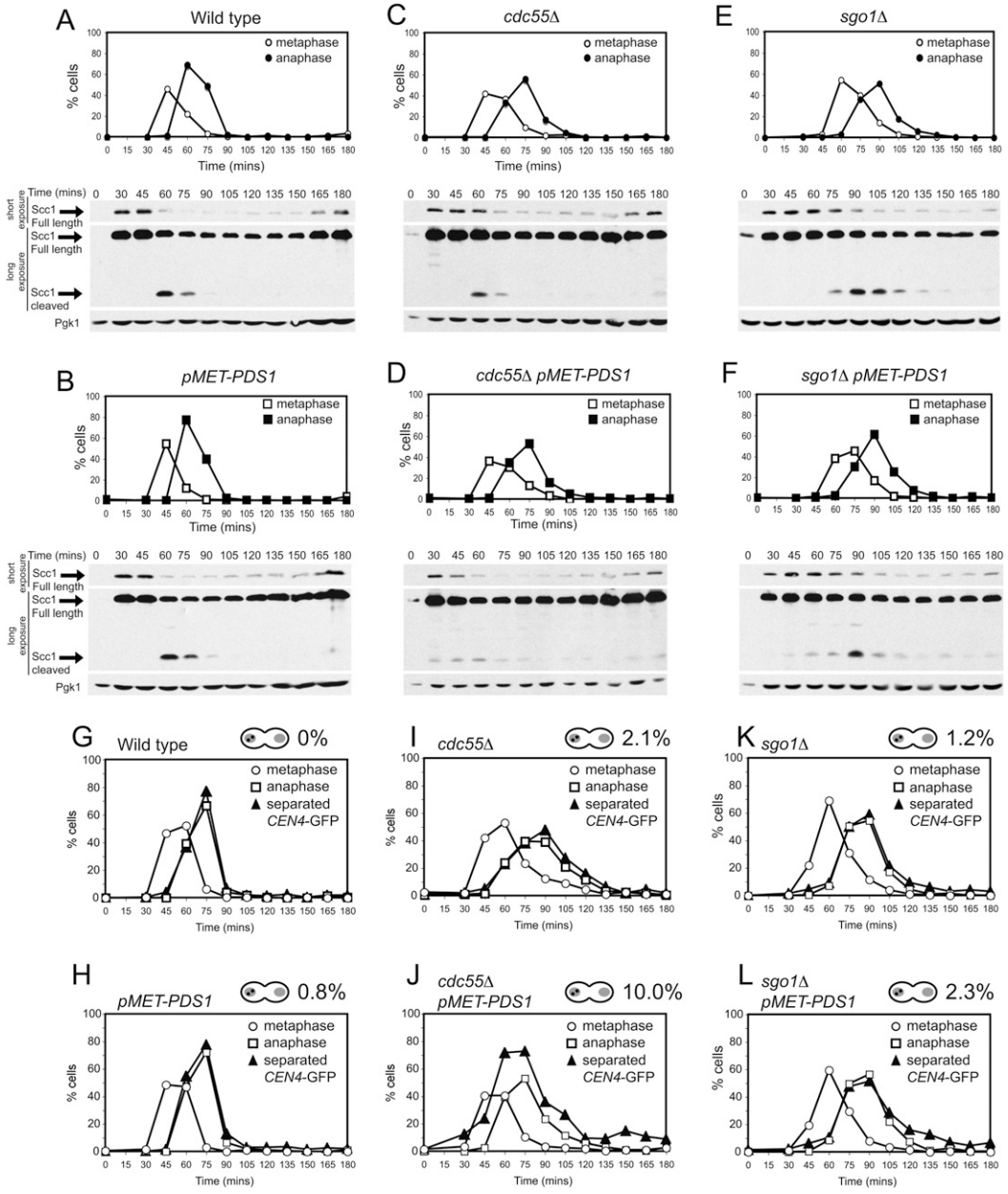

Figure 7. Loss of both *CDC55* and *PDS1* leads to premature cohesin cleavage, centromere separation, and aneuploidy. (*A–F*) Cohesin cleavage (*bottom* panels) and spindle morphology (*top* panels) in cells lacking *CDC55* or *SGO1* and depleted for *PDS1*. Strains of wild type (AM1145), *pMET-PDS1* (AM4429), *cdc55Δ* (AM4342), *pMET-PDS1 cdc55Δ* (AM4675), *sgo1Δ* (AM1474), and *pMET-PDS1 sgo1Δ* (AM4422) all carrying an *SCC1-6HA* fusion were grown in SC/−Met containing 2% glucose (SC/−Met/D) and arrested in G1 using α-factor. Cells were released into YEPD + 8 mM methonine and samples collected for anti-tubulin immunofluorescence and anti-HA Western blotting. A short and a long exposure of the same immunoblot are shown to allow both the decline in full-length Scc1 and the appearance of the unstable Scc1 cleavage product to be visualized. Pgk1 is shown as a loading control. (*G–L*) Sister centromeres separate prematurely leading to nondisjunction in cells lacking *CDC55* and depleted for *PDS1*. Strains of wild type (AM2812), *pMET-PDS1* (AM3665), *cdc55Δ* (AM4330), *pMET-PDS1 cdc55Δ* (AM4676), *sgo1Δ* (AM962), and *pMET-PDS1 sgo1Δ* (AM3865) all carrying *CENIV-GFP* were grown as described in *A–F*. The percentages of cells with metaphase or anaphase spindle morphology or with two separated GFP foci were scored. The percentage of large budded cells with divided nuclei in which two GFP dots were observed in the same nucleus is shown *above* the graph in each case.

after 60 min in *cdc55Δ* cells, although the decline in full-length Scc1 was less efficient. Interestingly, the decline in full-length cohesin was advanced in *cdc55Δ* cells that were also depleted for *PDS1* (Fig. 7D) and a low level of cleaved Scc1 was detected after only 30 min. These findings suggested the possibility that a subset of separase could be prematurely active in cells lacking both Cdc55 and Pds1. We also examined the consequences of loss of both *SGO1* and *PDS1* for cohesin cleavage. Although *sgo1Δ pds1Δ* double mutants are viable, they exhibit a synthetic growth defect that precludes reliable analysis of cell cycle progression; therefore, we again made use of the *pMET-PDS1* allele. Appearance of the Scc1 cleavage fragment was delayed to 75 min in *sgo1Δ* cells (Fig. 7E); however, a low level of cleaved Scc1 appeared after 45 min in *sgo1Δ pMET-PDS1* cells, although the decline of full-length Scc1 was not advanced (Fig. 7F). Therefore, Sgo1 may also play a minor role in the prevention of cohesin cleavage in cells lacking Pds1.

These findings prompted us to examine the functional consequences of depletion of *PDS1* in combination with *cdc55Δ* or *sgo1Δ* on chromosome segregation. For this purpose, we released cells with *CEN4-GFP* from a G1 block into methionine-containing medium and compared the timing of sister centromere separation to spindle morphology (Fig. 7G–L). Wild-type, *pMET-PDS1*, *cdc55Δ*, and *sgo1Δ* cells all separated sister *CEN4-GFP* signals simultaneously with the appearance of anaphase spindles, as expected. Separation of sister centromeres was also not significantly advanced in *sgo1Δ pMET-PDS1* cells, despite the premature appearance of cleaved Scc1 in these cells (Fig. 7F). Strikingly, however, *CEN4-GFP* signals split prior to the appearance of anaphase spindles in *cdc55Δ pMET-PDS1* cells, indicating premature loss of cohesion at *CEN4* (Fig. 7J). The outcome of this premature separation for the progeny was revealed by scoring the percentages of large budded cells with divided nuclei, in which both copies of *CEN4* had been partitioned to the same nucleus (Fig. 7G–L, percentages above graphs). A substantial degree (10%) of nondisjunction of *CEN4*-GFP was observed in *cdc55Δ pMET-PDS1* cells compared with the other strains tested (Fig. 7J). Missegregation of the other 15 chromosomes at a similar frequency would result in a level of aneuploidy that could account for the inviability of *cdc55Δ pMET-PDS1* cells.

We also tested the segregation of a chromosomal site just outside the centromere of chromosome V (*URA3-GFP*; ~35 kb away from the centromere) (Supplemental Fig. S10). *cdc55Δ pMET-PDS1* cells exhibited a less pronounced, precocious separation of *URA3-GFP* sequences, although this led to a similar degree of nondisjunction (8.8%) of chromosome V as for chromosome IV. These results suggest the interesting possibility that the precocious loss of sister chromatid cohesion in cells lacking both Pds1 and Cdc55 could be initiated at centromeres. Moreover, the finding that Pds1 and Cdc55 act redundantly to inhibit loss of sister chromatid cohesion suggests that Cdc55 and Pds1 work together to restrain separase activity.

Discussion

Cdc55 is a separase inhibitor

Separase is universally required to trigger chromosome segregation through its role in cohesin cleavage (Uhlmann et al. 2000; Wirth et al. 2006). Due to the irreversible and vital nature of this event, cells have evolved redundant modes of regulation. Securin, an inhibitory chaperone for separase, is a key player, although it is not essential for cell growth in several systems, including humans (Alexandru et al. 1999; Jallepalli et al. 2001; Mei et al. 2001). Prevention of cohesin subunit phosphorylation, although strongly inhibitory for cleavage of meiotic cohesins, plays a lesser role in mitosis and causes only a modest delay in cohesin cleavage (Alexandru et al. 2001; Clyne et al. 2003; Lee and Amon 2003). Our analysis of Sgo1 function in mitotic cells has uncovered an additional level of regulation: separase inhibition by $PP2A^{Cdc55}$. Several lines of evidence support our conclusion that Cdc55 is a separase inhibitor: First, overexpression of *CDC55* prevents the cleavage of separase substrates in a securin-independent manner. Second, deletion of *CDC55* allows separase activation in *SGO1*-overexpressing cells. Third, cohesin is ectopically cleaved in *CDC55*-depleted meiotic cells where cohesin phosphorylation is prevented. Finally, we showed that Cdc55 and Pds1 play redundant roles in inhibiting precocious cohesin cleavage and sister chromatid separation.

How does $PP2A^{Cdc55}$ inhibit separase? Cdc55 and separase are known to form a complex in vivo, and $PP2A^{Cdc55}$ phosphatase activity is maximal in metaphase but declines as cells enter anaphase (Queralt et al. 2006). Human separase has also been shown to interact with B and B′ regulatory subunits of PP2A (Gil-Bernabe et al. 2006; Holland et al. 2007). Therefore, $PP2A^{Cdc55}$ could inhibit separase activity by direct dephosphorylation. However, the regulatory interactions between Cdc55 and separase are likely to be complex since a reciprocal role for separase in down-regulating $PP2A^{Cdc55}$ has been described (Queralt et al. 2006). Therefore, Cdc55 and separase could constitute a regulatory feedback mechanism that coordinates the initiation of chromosome segregation with mitotic exit (Fig. 2A). Such a mechanism could explain an apparent paradox presented by our findings: While *CDC55* or *SGO1* overexpression results in securin stabilization, securin is not required for the block to anaphase. Perhaps inhibition of separase by $PP2A^{Cdc55}$ is in itself sufficient to prevent securin degradation.

Anaphase onset upon cleavage of a minor pool of cohesin

Although deletion of *CDC55* largely abrogates the metaphase delay in *SGO1*-overexpressing cells, separase does not appear to be completely active in these cells because securin degradation is retarded and cohesin cleavage is inefficient. One possible explanation is that overexpressed Sgo1 targets $PP2A^{Rts1}$ to cohesin and prevents its phosphorylation, making it a poor substrate for cleavage (Brar et al.

2006; Kitajima et al. 2006; Riedel et al. 2006). This uncleaved cohesin is not, however, localized to chromosomes, explaining why it is unable to hold chromosomes together. Therefore, *CDC55* is required to prevent cleavage of a minor pool of cohesin, which appears to be the most functionally relevant in generating intersister linkages. Interestingly, the findings of Tang and Wang (2006) have suggested that *CDC55* is required to prevent sister centromere separation even under conditions where Pds1 is stable. Furthermore, the premature loss of cohesion in cells lacking both *PDS1* and *CDC55* is initiated at the centromere. Perhaps *CDC55* is especially important for preventing the cleavage of cohesins in this region that, due to their proximity to the site of microtubule attachment, could be the most critical in holding chromosomes together.

Cdc55 carries out the cellular functions of Sgo1

Previous studies showed that Sgo1 both senses a lack of tension between sister kinetochores in mitosis and protects centromeric cohesion during meiosis, but the underlying mechanisms were unresolved. We provided evidence that $PP2A^{Cdc55}$-dependent separase inhibition is at least partially responsible for both of these activities of Sgo1. This pathway contributes to the prevention of cohesin cleavage during meiosis I and operates alongside the predominant pathway of cohesin dephosphorylation through $PP2A^{Rts1}$ recruitment. Therefore, Sgo1 acts on both the substrate (cohesin) and enzyme (separase), through two different PP2A regulatory subunits, thereby ensuring the inhibition of cohesin cleavage at centromeres during meiosis I. Our data also support the notion that $PP2A^{Cdc55}$ carries out the functions of Sgo1 in tension-sensing. Previous studies in fission yeast suggested that Sgo2 promotes sister kinetochore biorientation through localization of the Aurora kinase, which is known to destabilize inappropriate kinetochore–microtubule attachments (Kawashima et al. 2007; Vanoosthuyse et al. 2007). This leads to activation of the canonical SAC, resulting in checkpoint arrest due to securin stabilization (Pinsky et al. 2006). However, budding yeast Sgo1 does not obviously regulate Aurora kinase (Ipl) localization (Kiburz et al. 2008) and, moreover, Sgo1 can prevent anaphase onset independently of securin or Aurora kinase. Instead, our data support a model whereby Sgo1 and Cdc55 work in a common checkpoint pathway that senses and responds to a lack of tension between sister kinetochores in a manner distinct from the canonical SAC. We speculate that a failure to inhibit separase activity in *cdc55Δ* and *sgo1Δ* mutants in response to a lack of tension causes cleavage of its substrates and the observed release of Cdc14 from the nucleolus. How then does Sgo1 instruct $PP2A^{Cdc55}$ to inhibit separase in response to a lack of tension? One possibility is that $PP2A^{Cdc55}$, like $PP2A^{Rts1}$ (Riedel et al. 2006), directly interacts with Sgo1. However, we did not detect an interaction between Sgo1 and Cdc55, nor did we find Cdc55 associated with the pericentromere (data not shown). We therefore postulate the existence of intermediary proteins that function in the separase regulatory network.

Surveillance mechanisms converge on Cdc55

Our securin depletion experiments suggest that, in contrast to Cdc55, Sgo1 plays only a minor role in restricting separase activation in a normal cell cycle. We suggest that Cdc55 is a master cell cycle regulator that integrates signals from different cellular cues, one of which is a lack of tension through Sgo1. In support of this idea, *CDC55* is required to prevent anaphase onset upon activation of the morphogenesis checkpoint (Chiroli et al. 2007) or overexpression of the DNA replication factor and mitotic regulator, *CDC6* (Boronat and Campbell 2007). Therefore, multiple surveillance mechanisms could converge on Cdc55, thereby ensuring the strict order of events in the cell cycle.

In summary, we uncovered an additional mode of cohesin regulation and identified Cdc55 as a separase inhibitor. Our findings demonstrate that Sgo1 performs its functions in monitoring sister kinetochore biorientation and in the protection of cohesin during meiosis, at least partially through Cdc55. Intriguingly, both of these situations in which Sgo1 performs its essential functions are characterized by a lack of tension across sister kinetochores. The exertion of tension at sister kinetochores has been proposed to inactivate Sgo1's cohesin protection function (Gomez et al. 2007; Lee et al. 2008). Perhaps the absence of tension is the universal stimulus to which Sgo1 responds by locally eliciting the $PP2A^{Cdc55}$-dependent inhibition of separase. An important challenge for the future will be to understand how $PP2A^{Cdc55}$-dependent separase inhibition is integrated with other checkpoint mechanisms that regulate anaphase onset.

Materials and methods

Strains, plasmids, and growth conditions

The strains used in this study are described in Supplemental Table S1 and are all derivatives of W303, except for the experiments shown in Figure 6 where SK1 was used. The *pGAL-SGO1* and *pGAL-CDC55* strains were generated by integration of plasmids AMp37 and AMp649, in which *SGO1* and *CDC55* are cloned downstream from the *pGAL1-10* promoter, into the *URA3* and *TRP1* loci, respectively. A *pGAL-SGO1-MYC* plasmid (AMp41) was similarly constructed following amplification of *SGO1-9MYC* from strain AM905. The *cdc55Δ*, *rts1Δ*, *pph22Δ* and *pph21-L369Δ* alleles were generated by one-step PCR using pFA6a-kanMX6 (Longtine et al. 1998). The *pCLB2-3HA-CDC55* allele was generated by the PCR-based method described in Longtine et al. (1998), using a template described previously (Lee and Amon 2003). The *pMET3-PDS1* construct was generated similarly, using a template (B. Lee and A. Amon, unpubl.) in which the *pMET3* promoter is inserted downstream from the KanMX cassette in pFA6akanMX6 (Longtine et al. 1998). Growth conditions for individual experiments are given in the figure legends. All mitotic time courses were performed at room temperature unless otherwise stated. Glucose, raffinose, and galactose were all used at 2%. To depolymerize microtubules,

a mixture of nocodazole (15μg/mL) and benomyl (30 μg/mL) was used. For depletion of genes under the methionine-repressible promoter *pMET3*, cells were grown overnight in minimal medium lacking methionine (S/−met) and *pMET3* was subsequently repressed by growth in rich medium containing 8 mM methionine. Methionine was added again to 4 mM every hour. Meiotic time courses were performed at 30°C as described in Marston et al. (2003).

Microscopy

Indirect immunofluorescence was performed as described by Visintin et al. (1999). Chromosome spreading was performed using the methods of Nairz and Klein (1997) and Loidl et al. (1998). Tubulin was visualized using an anti-rat antibody at a dilution of 1:50 and an anti-rat FITC antibody at a dilution of 1:100. For detection of 3HA-Cdc14, a mouse HA antibody was used at a dilution of 1:150 followed by an anti-mouse Cy3 conjugate at a dilution of 1:500. For detection of Pds1-18Myc, a mouse anti-Myc antibody was used at a dilution of 1:250 followed by an anti-mouse Cy3 antibody at a dilution of 1:1000. GFP-labeled chromosomes were visualized as detected by Klein et al. (1999). Samples were analyzed on a Zeiss Axioplan 2 microscope and images were grabbed using a Hamamatsu camera operated through Axiovision software. Spindle measurements were performed using Image Pro software.

Immunoblot analysis

Samples for immunoblot analysis were fixed in 5% TCA and cell pellets were washed once with acetone. Cells were lysed in 50 mM Tris (pH 7.5), 1 mM EDTA, and 50 mM DTT containing protease inhibitors, with glass beads, and boiled in 1× sample buffer. Immunoblot analysis was performed using a mouse anti-Myc 9E10 antibody at 1:1000 dilution, a mouse HA-11 antibody at 1:1000 dilution, or a mouse Pgk1 antibody at 1:5000. Kar2 antibody (Rose et al. 1989) was used at a dilution of 1:10,000.

ChIP

ChIP was performed as described by Kiburz et al. (2005). For ChIP of HA-tagged proteins, the 12CA5 antibody was used, and for ChIP of Myc-tagged proteins, the 9E10 antibody was used. Semiquantitative PCR analysis was performed on immunoprecipitates, samples were run on ethidium bromide-stained agarose gels, and the signal was quantified as described in the figure legends using Image J software. Each experiment was repeated at least twice and a representative experiment is shown. Sequences of primers are available on request.

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